

## Distribution of Prolylhydroxyproline and Its Metabolites after Oral Administration in Rats

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Received September 27, 2011; accepted December 20, 2011; published online December 22, 2011

Prolylhydroxyproline (Pro-Hyp), which is derived from collagen hydrolysate, has been shown to be beneficial for skin and joint health. However, little is known about the distribution of Pro-Hyp in these tissues. In the present study, we investigated the biodistribution of orally administered [<sup>14</sup>C]Pro-Hyp in rats. Whole-body autoradiography at 30 min after administration of [<sup>14</sup>C]Pro-Hyp showed that radioactivity is widely distributed in tissues including skin and articular cartilage, with the highest level of radioactivity observed in the gastric and intestinal walls. Incorporation of radioactivity into cells known to respond to Pro-Hyp such as dermal fibroblasts, synovial cells, chondrocytes, osteoblasts, and osteoclasts was observed. The chemical form of [<sup>14</sup>C]Pro-Hyp-derived radioactivity detected in the tissues was investigated by thin layer chromatography. The radioactive constituents in cartilage extract were two proline-modified peptides (56%), intact Pro-Hyp (5%), and two nonpeptide metabolites (28%). Similar results were obtained for skin and bone marrow. Plasma analysis at 3 to 30 min post-dose suggested that the majority of Pro-Hyp is modified in its proline residue by a first-pass effect without peptide bond hydrolysis. In conclusion, we demonstrated that Pro-Hyp is partly distributed in observed tissues including skin and cartilage in its intact form, which might be responsible for its biological functions.

**Key words** prolylhydroxyproline; distribution; collagen; autoradiography

Collagen is one of the major constituents of the extracellular matrix and is found in cartilage, bone, tendons, skin, blood vessels, and other connective tissues. Gelatin, the heat-denatured form of collagen, is widely used in pharmaceuticals, films, and foods. Gelatin is further hydrolyzed with enzymes or acid to improve its solubility. This product is called collagen peptide, collagen hydrolysate, and gelatin hydrolysate. Intake of collagen hydrolysate has been believed to improve joint disorders and skin damage. Randomized, double-blind, placebo-controlled trials were recently conducted. Intake of 10 g/d of collagen hydrolysate for 6 months significantly reduced knee pain in osteoarthritis patients.<sup>1</sup> Significant increase in moisture content of the cheek stratum corneum after ingesting 10 g/d collagen hydrolysate for 4 weeks was reported, although the efficacy was limited to volunteers >30 years of age.<sup>2</sup> Animal studies also supported these positive effects of collagen hydrolysate.<sup>3–6</sup> However, the mechanisms underlying these effects have not been fully elucidated.

Orally administered collagen is digested by a combination of pepsin and pancreatic juice. The digestive products are absorbed in the small intestine<sup>7</sup> via the proton-coupled oligopeptide transporter PEPT1 for di- and tripeptides<sup>8</sup> or via paracellular transport for high-molecular-weight peptides.<sup>9</sup> In human blood and urine, collagen-derived Hyp-containing peptides were observed for several hours after ingestion.<sup>10</sup> Oesser *et al.* reported that part of the absorbed peptides accumulates in cartilage, although compositions of the peptides remain unclear.<sup>9</sup> The amino acid sequences of collagen-derived peptides in human blood were recently determined. The major peptide was prolylhydroxyproline (Pro-Hyp), and the others were Pro-Hyp-Gly, Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp, Ser-Hyp-Gly, Leu-Hyp, Ile-Hyp, and Phe-Hyp.<sup>11,12</sup>

As a key molecule in collagen hydrolysate ingestion for treatment of joint disorders and skin damage, biological activities of Pro-Hyp have been investigated by *in vitro* and

*in vivo* studies.<sup>13–17</sup> Briefly, oral administration of Pro-Hyp suppressed some skin damage in UV-irradiated hairless mice, such as skin barrier destruction and skin thickening.<sup>13</sup> An *in vitro* study showed that Pro-Hyp stimulates cell proliferation and hyaluronic acid synthase 2 gene expression in dermal fibroblasts.<sup>14,15</sup> In terms of joint health, Pro-Hyp inhibited phosphorus-induced degradation of mice cartilage, including the loss of chondrocytes and thinning of the articular cartilage layer.<sup>16</sup> In an *in vitro* study, the authors also reported that Pro-Hyp regulates the differentiation of chondrocytes into their mineralized form and stimulates glycosaminoglycan synthesis in chondrocytes. Furthermore, Ohara *et al.* reported that Pro-Hyp also enhances hyaluronic acid synthesis in cultured synovial cells.<sup>17</sup> These reports suggest that Pro-Hyp is most likely to contribute to efficacy of collagen hydrolysate ingestion. Generally, the bioactive peptide should be distributed in tissues and cells at least partly in its active form to exert its biological activities.<sup>18</sup> However, the distribution of orally administered Pro-Hyp remains unclear.

In this study, we investigated the biodistribution of Pro-Hyp after oral administration in rats using radioactive tracer techniques.

### MATERIALS AND METHODS

**Radiolabeled Materials** [<sup>14</sup>C]Pro-Hyp (radiochemical purity >98%, specific activity 27.0 mCi/mmol) and [<sup>14</sup>C]Pro (radiochemical purity 99%, specific activity 256 mCi/mmol) were purchased from Muromachi Yakuhin Kaisha Ltd. (Muromachi, Tokyo, Japan). [<sup>14</sup>C]Pro-Hyp was labeled with [<sup>14</sup>C] in its Pro moiety.

**Animals** Six-week-old male Sprague–Dawley rats (Charles River Laboratories Japan Inc., Shin-Yokohama, Kanagawa, Japan) were conditioned for 1 week in a temperature- and humidity-controlled room with free access to

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food (CE2; CLEA Japan Inc., Higashiyama, Tokyo, Japan) and water. The dark/light cycle was 12/12h. This study was performed in accordance with the Japanese Guidelines for Nonclinical Pharmacokinetic Studies.<sup>19)</sup>

**Whole-Body Autoradiography** A radiolabeled compound ( $22\mu\text{Ci}$ ) was orally administered to rats by a stomach tube after an 18-h fast. The rats were sacrificed at 0.5 and 24 h after administration. The animals were frozen in liquid nitrogen. Thin sections of the frozen samples were prepared using a Cryomacrocut (Leica Instruments GmbH, Nussloch, Germany) and then freeze-dried. The cryosections were exposed to an imaging plate (Fuji Photo Film Co., Ltd., Nishiazabu, Tokyo, Japan) for an appropriate period. Autoradiographs were obtained and quantified by BAS2000 (Fuji Photo Film Co., Ltd.). The radioactivity in the region of interest was expressed as photo-stimulated luminescence (PSL) minus background (BG) per square millimeter and was graded as low ( $1\text{--}10$  (PSL-BG)/ $\text{mm}^2$ ), moderate ( $10\text{--}100$  (PSL-BG)/ $\text{mm}^2$ ), or high ( $>100$  (PSL-BG)/ $\text{mm}^2$ ).<sup>20)</sup>

**Microautoradiography** [ $^{14}\text{C}$ ]Pro-Hyp ( $75.9\mu\text{Ci}/\text{rat}$ ) was orally administered to rats by a stomach tube after an 18-h fast. After 30 min, rats were sacrificed and their skin and femurs were collected. The fixed paraffin-embedded tissue was prepared and then sliced by a microtome. Paraffin was removed from the sample. The slice was coated with nuclear track emulsion (NTB; Kodak, New York, U.S.A.) and exposed for  $>1$  month. After exposure, the slice was developed and stained with hematoxylin and eosin. The developed silver grains (black spots) overlying the tissue were observed under a microscope.

**Thin Layer Chromatography** Rats administered [ $^{14}\text{C}$ ]Pro-Hyp ( $75.9\mu\text{Ci}$ ) were sacrificed, and their skin and bone marrow were collected at 0.5, 6, 24, and 72 h. Cartilage sample was collected from rat administered [ $^{14}\text{C}$ ]Pro-Hyp ( $222.6\mu\text{Ci}$ ) to obtain detectable levels of radioactivity at 0.5 h post-dose. The sample was mixed with a 10-fold volume of purified water and then homogenized. After centrifugation, the supernatant was spotted onto TLC plate (TLC Silica Gel 60 F264; Merck, Darmstadt, Germany) with standards (Wako Pure Chemical Ind., Ltd., Doshu-cho, Osaka, Japan). Development was carried out with two solvent systems of 2-propanol/ $\text{NH}_4\text{OH}$  (3 : 1) and *n*-BuOH/EtOH/water (2 : 2 : 1). After development, the plate was exposed to an imaging plate (BAS-III; Fuji Photo Film Co., Ltd., Tokyo, Japan) for an appropriate period. Autoradiographs of TLC profiles were obtained and quantified by BAS2000.

Peripheral blood was collected in heparin tubes at 3, 6, 10, and 30 min after oral administration of [ $^{14}\text{C}$ ]Pro-Hyp ( $69.0\mu\text{Ci}/\text{rat}$ ). Plasma was obtained by centrifugation ( $4^\circ\text{C}$ , 3000 rpm, 15 min). After filtration (Nanosep 3K OMEGA; Pall Corp., Ann Arbor, Michigan, U.S.A.), the filtrate was spotted onto TLC plates with standards. Development and analysis were performed as described. Spots of nonradioactive standards were detected by spraying the developed plate with 0.2% ninhydrin solution in ethanol with the exception that iodine was used for detection of  $\alpha$ -ketoglutaric acid and diketopiperazine of Pro-Hyp.

## RESULTS

### Tissue and Organ Distribution of Radioactivity after

**Oral Administration of [ $^{14}\text{C}$ ]Pro-Hyp** Whole-body autoradiography was performed to visualize tissue and organ distribution of orally administered Pro-Hyp in rats. Thirty minutes after oral administration of [ $^{14}\text{C}$ ]Pro-Hyp, radioactivity was widely distributed in the rat (Fig. 1A). The highest level of radioactivity was observed in the gastric and intestinal walls. Moderate radioactivity was observed in the liver, spleen, kidney, pancreas, bone marrow, skin, articular cartilage, and glands. Figure 1B shows whole-body autoradiographs at 24 h after [ $^{14}\text{C}$ ]Pro-Hyp administration. Compared with that at 30 min, radioactivity was slightly decreased, while the distribution was similar with the following exceptions. Accumulation of radioactivity was observed in alimentary canal mucosa, including oral cavity and gastrointestinal mucosa. Radioactivity could not be detected in stomach contents or eyes.

As a comparison, autoradiography of [ $^{14}\text{C}$ ]Pro was also performed. Figure 1C shows the distribution of radioactivity at 30 min after [ $^{14}\text{C}$ ]Pro administration. Gastrointestinal contents showed the highest radioactivity. A moderate level of activity was observed in the liver, spleen, kidney, small intestine, bone marrow, skin, and glands. Similar distribution was observed at 24 h after administration except for the disappearance of radioactivity in the stomach (Fig. 1D).

These results demonstrate several differences in distribution between Pro-Hyp and Pro as follows. Radioactivity from gastrointestinal contents at 30 min after oral administration of [ $^{14}\text{C}$ ]Pro was higher than that after oral administration of [ $^{14}\text{C}$ ]Pro-Hyp. Furthermore, gastrointestinal mucosa was specifically labeled by [ $^{14}\text{C}$ ]Pro-Hyp. In bone, radioactivity was observed mainly in the periosteum for [ $^{14}\text{C}$ ]Pro but in bone marrow for [ $^{14}\text{C}$ ]Pro-Hyp. Skin was homogeneously labeled by [ $^{14}\text{C}$ ]Pro-Hyp, whereas accumulation in sweat glands and hair roots was observed for [ $^{14}\text{C}$ ]Pro.

**Cellular Uptake of Radioactivity after Oral Administration of [ $^{14}\text{C}$ ]Pro-Hyp** To evaluate whether radioactivity derived from [ $^{14}\text{C}$ ]Pro-Hyp administration is incorporated into cells, microautoradiography of skin and femur sections obtained at 30 min after administration was performed.

In skin, silver grains within fibroblasts sparsely distributed in the dermis and epidermal cells were detected (Fig. 2A). Interestingly, silver grains accumulated in the epidermis rather than in the dermis. In the femur, silver grains were incorporated into chondrocytes (Fig. 2B) and synovial cells (Fig. 2C). Bone did not show silver grain accumulation, whereas clusters of silver grains were frequently observed in the bone marrow (Fig. 2D). In the bone marrow, silver grains were found in osteoblasts, stem cells, and hematopoietic cells. Osteoclasts also contained silver grains (Fig. 2E).

**TLC Analysis of Radioactive Compounds in Tissues** Our findings point out the possibility that Pro-Hyp was distributed in the observed tissues and cells in the intact or metabolized peptide form. To evaluate this possibility, we analyzed the radioactive compounds present in the tissues obtained at 30 min after oral [ $^{14}\text{C}$ ]Pro-Hyp administration using thin layer chromatography.

Figure 3A shows a typical TLC autoradiograph of a cartilage sample developed with 2-propanol/ $\text{NH}_4\text{OH}$  (3 : 1) solution. A dim but detectable band (indicated as band 1 in the figure) of intact Pro-Hyp was observed. This result indicates that Pro-Hyp was distributed in cartilage partly in the intact form, but

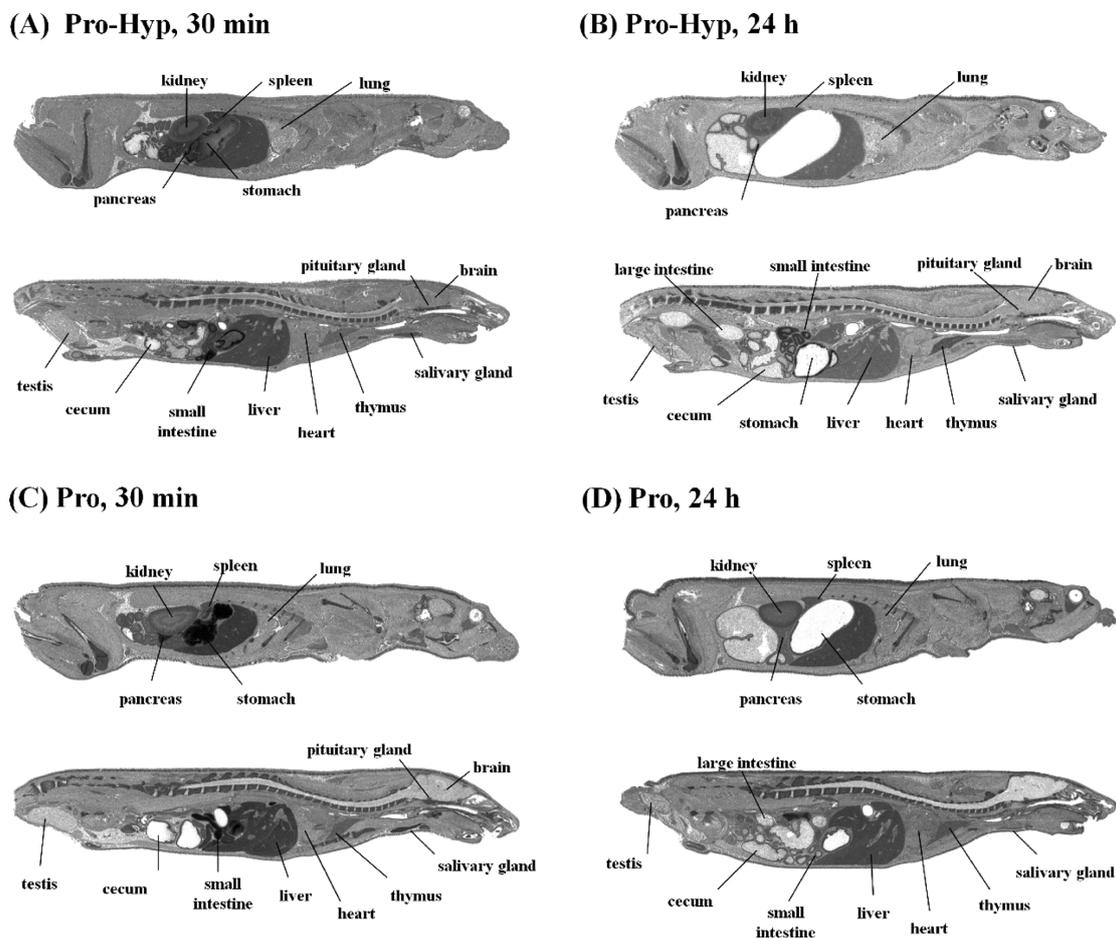


Fig. 1. Whole-Body Autoradiographs of Rats after Single Oral Administration

Autoradiographs of orally administered  $^{22}\mu\text{Ci}$  radioactive tracers to rats were obtained. Upper autoradiographs are obtained from the dorsoventral section, including the kidney. Lower is the dorsoventral mesion section. Black represents the presence of radiocarbon. (A,B) Autoradiographs of rats at 30 min (A) and 24 h (B) after oral administration of [ $^{14}\text{C}$ ]Pro-Hyp. (C,D) Autoradiographs of rats at 30 min (C) and 24 h (D) after oral administration of [ $^{14}\text{C}$ ]Pro.

mainly in modified forms. A trace amount of Pro, expected to be a plausible degradation product of Pro-Hyp, was detected by the use of two solvents (data not shown). Only a few radiocarbons attached to Pro indicates that the majority of Pro-Hyp was modified in its proline residue without peptide bond hydrolysis. To confirm the presence of the proline-modified peptides, the cartilage sample was hydrolyzed by 6N HCl for 24 h at 110°C. Compared with the nonhydrolyzed profile, two bands (bands 2 and 5) other than intact Pro-Hyp disappeared after the hydrolysis almost without any increase in radioactivity of the Pro-corresponding position. This result indicates that two proline-modified peptides were present in this tissue. The ratio of each to the total radioactivity in the TLC lane is represented in Fig. 3B. The sum of three peptide form compounds containing intact Pro-Hyp (4.9%) reached 60.6% in cartilage at 30 min after oral administration. These results demonstrate the peptidase-resistance of Pro-Hyp. Similar results were obtained from skin and bone marrow samples (Fig. 3B). At time points beyond 6 h, we could not analyze the TLC data because of the low radioactivity of samples for quantification. We analyzed other nonpeptide metabolites (bands 3 and 4) using various standards including intermediates (Gly, Ser, Gln, Glu, Asp, and  $\alpha$ -ketoglutaric acid) of the metabolic pathway of Pro and Hyp and diketopiperazine of Pro-Hyp as an experimental byproduct.<sup>21)</sup> This analysis was

also performed with hydrolyzed samples to sequence the proline-modified peptides (bands 2 and 5). However, the metabolites did not clearly correspond to such standards. These results demonstrate that orally administered Pro-Hyp is partly distributed in the observed tissues in intact form, but mainly in proline-modified peptide forms.

These findings raise a question. When and where is the proline residue of Pro-Hyp modified? To address the stated question, we analyzed peripheral blood collected at 3, 6, 10, and 30 min after oral administration (Fig. 4A). TLC profiles of plasma samples were similar to that of the cartilage sample (Fig. 3A) except for the occurrence of a smear band (band 1) with the highest radioactivity neighboring the Pro-Hyp position. Radioactivity of bands 3 and 5 increased with increasing time. Band 1 largely disappeared at 30 min. Acid hydrolysis revealed that the constituent of the smear band is mainly a proline-modified peptide because radioactivity disappeared after hydrolysis (Fig. 4B). These results suggest that orally administered Pro-Hyp was largely modified in its proline residue before entering the blood circulation, probably because of a first-pass effect, and these metabolites and Pro-Hyp were then distributed in the tissues.

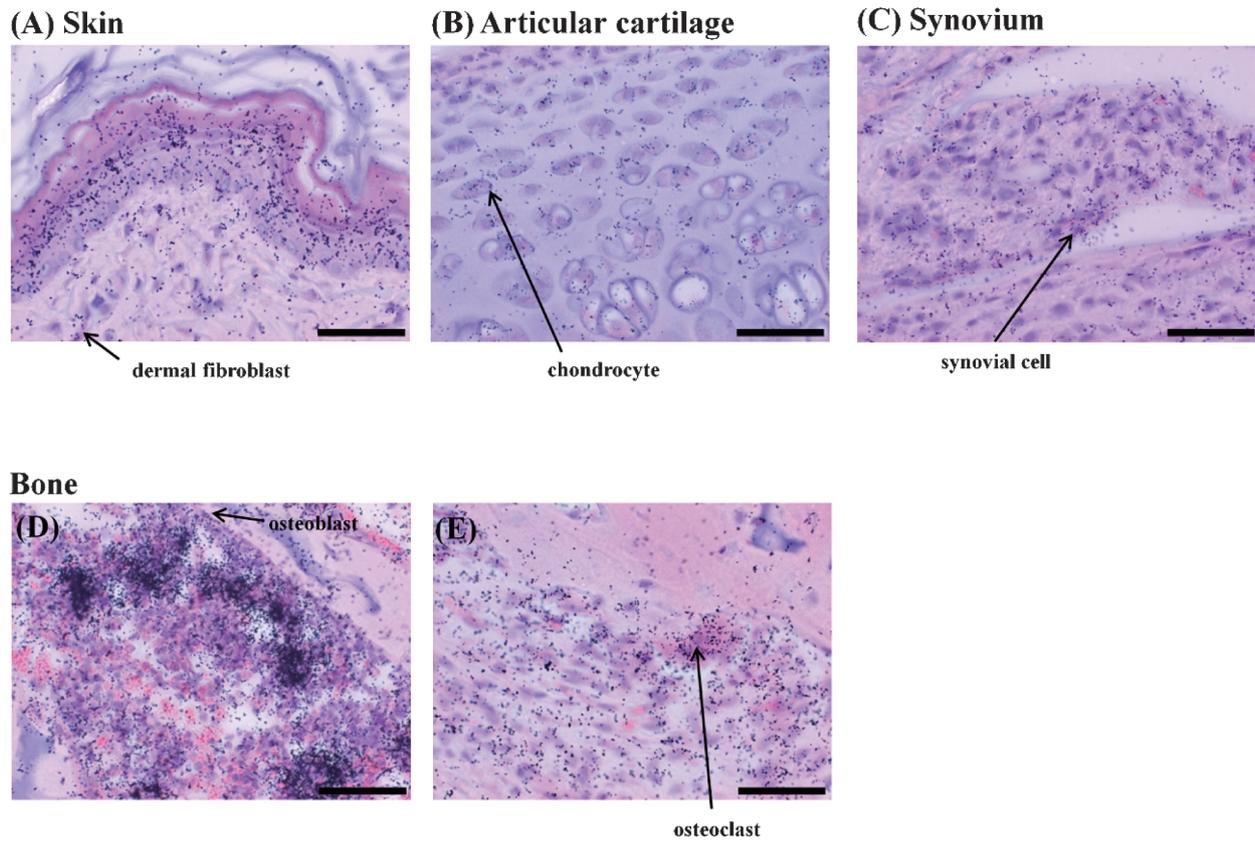


Fig. 2. Autoradiographs of Rat Tissue Sections Obtained at 30min after Oral Administration of [<sup>14</sup>C]Pro-Hyp

Tissues were stained by hematoxylin and eosin. (A) skin, (B) articular cartilage, (C) synovium, and (D,E) bone. Radiocarbon is represented as black particles of reduced silver grains. The arrow indicates incorporation of radiocarbon into cells. Bar=50µm.

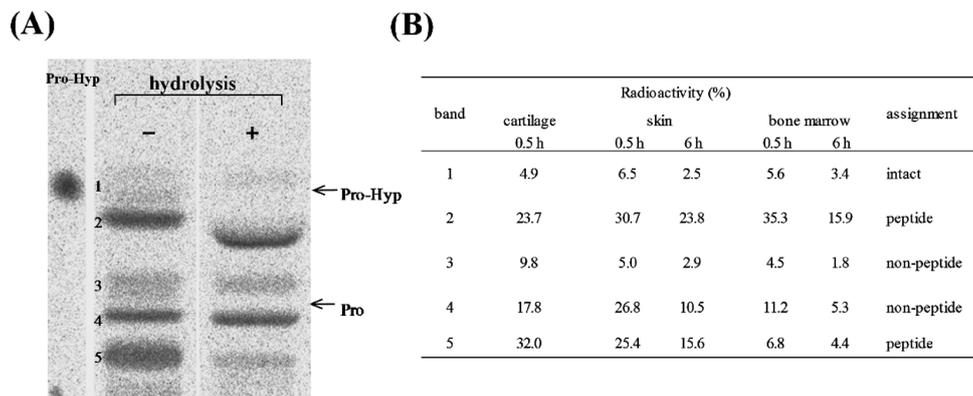


Fig. 3. TLC Analysis of Radioactive Compositions Present in Tissues at 30min after Oral Administration of [<sup>14</sup>C]Pro-Hyp

(A) TLC profile of a cartilage sample. The arrow shows the corresponding spot of Pro-Hyp and Pro. The Arabic numerals denote band numbers. The right lane shows the profile of the cartilage hydrolysate sample. (B) Quantification of radioactive compounds in tissues. Percentages were calculated by dividing each radioactivity by total detected radioactivity.

DISCUSSION

We studied the biodistribution of orally administered [<sup>14</sup>C] Pro-Hyp, a collagen-derived bioactive peptide, in rats using autoradiography. The main objective in this study was to evaluate the uptake and chemical form of the absorbed [<sup>14</sup>C] Pro-Hyp in tissues.

Whole-body autoradiography of [<sup>14</sup>C]Pro-Hyp showed wide distribution of radioactivity in rat at 30min post-dose. Incorporation of the radioactivity at moderate levels into skin and cartilage was observed. This nonspecific but clear distri-

bution of the radioactivity in skin and cartilage was similar to that of Gly-Pro-Hyp, which is known as a collagen-derived precursor of Pro-Hyp.<sup>22,23)</sup> Compared with autoradiography of [<sup>14</sup>C]Pro, gastrointestinal contents after [<sup>14</sup>C]Pro-Hyp administration showed lower radioactivity. This result indicates rapid absorption of dipeptides compared with amino acids, as previously reported for Gly-Gly.<sup>24)</sup> Furthermore, differences in distribution were observed in skin, bone, and gastrointestinal mucosa. These results described above demonstrate that orally administered [<sup>14</sup>C]Pro-Hyp is absorbed and distributed in the observed tissues in peptide form. Furthermore, we showed

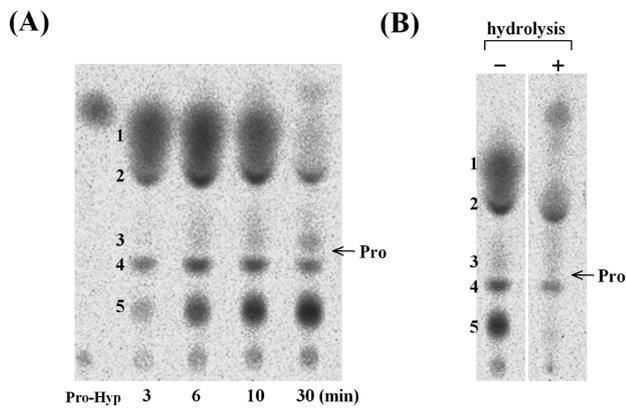


Fig. 4. TLC Analysis of Radioactive Compositions Present in Plasma at 3, 6, 10, and 30 min after Oral Administration of [ $^{14}\text{C}$ ]Pro-Hyp

The arrow shows the corresponding spot of Pro. The Arabic numerals denote band numbers. (A) TLC profile of plasma sample. (B) TLC profile of plasma collected at 10 min post-dose before (left) and after (right) hydrolysis.

the long-term, whole-body retention of radioactivity observed at 24 h post-dose of [ $^{14}\text{C}$ ]Pro-Hyp and the cellular uptake of radioactivity in dermal fibroblasts, epidermal cells, chondrocytes, synovial cells, osteoblasts, and osteoclasts using microautoradiography. These results might be important to elucidate its functions in terms of daily intake.

We then analyzed radioactive constituents in blood and tissue extracts by TLC to confirm their chemical forms. TLC analysis revealed the presence of peptide and nonpeptide metabolites as follows. Pro-Hyp entered the blood circulation in the form of three proline-modified peptides, intact Pro-Hyp, and two nonpeptide compounds with negligible amounts of Pro. One of the modified peptides disappeared during 30 min of circulation. These results are supported by a previous study. Liu *et al.* reported that the amount of absorbed intact Pro-Hyp following Pro-Hyp injection to the isolated perfused rat intestine system was lower than that of total peptide form compounds.<sup>25)</sup> The amount of absorbed free Hyp was also reported to be extremely low. These results indicate the peptidase-resistance of Pro-Hyp linkage<sup>26)</sup> and the presence of the modified form of Pro-Hyp. Next, Pro-Hyp was partly distributed in the observed tissues in its intact form (about 5%), but mainly in two proline-modified peptides (about 50%) at 30 min after oral administration. Considering that >80% of subcutaneously administered [ $^3\text{H}$ ]Pro-Hyp retained its intact form and was excreted in urine,<sup>26)</sup> these results may reflect low bioavailability of Pro-Hyp. The relatively low bioavailability was also reported.<sup>27)</sup> About 7% of ingested Pro-Hyp was absorbed and excreted in its intact form, although the blood level of Pro-Hyp was too low for detection. In contrast, in humans, a high concentration of Pro-Hyp in peripheral blood after collagen hydrolysate ingestion was reported.<sup>28)</sup> The relatively high bioavailability of Hyp-containing peptides was explained as efficient reabsorption *via* peptide transporters (PEPT-1 and PEPT-2) in the kidney and high resistance to peptidases.<sup>29)</sup> The proline-modified peptides that we observed have not been identified in previous studies.<sup>11,12)</sup> This raises novel possibilities about the effects of collagen hydrolysate or Pro-Hyp as follows. First, under equivalent ingestion of collagen hydrolysate, a large amount of bioactive Pro-Hyp in humans leads to higher effectiveness in terms of skin and joint health compared with rats. Second, the proline-modified

peptides are absent in humans and work instead of Pro-Hyp in rats. Third, the proline-modified peptides are also present in humans and work together with Pro-Hyp. Our findings regarding modification of Pro-Hyp also suggest that the peptide absorption reported previously was underestimated because of the modified proline nature of the metabolites during the separation process. Further investigation of the modified peptides in terms of their structures and biological activities will be required.

In summary, the present study demonstrates that orally administered Pro-Hyp was distributed in the observed tissues and cells, in which Pro-Hyp is expected to exert its biological effects. Furthermore, a large amount of proline-modified peptides compared with intact Pro-Hyp was detected in our study. Further investigation of proline-modified peptides will be required to elucidate the exact mechanism of the beneficial effect of dietary Pro-Hyp and collagen hydrolysate on skin and joint health.

**Acknowledgments** We are grateful to Dr. A. Shigematsu of the Institute of Whole Body Metabolism for valuable discussions. We also thank to Dr. K. Sato of Kyoto Prefectural University and Dr. Y. Shigemura of Osaka Yuhigaoka Gakuen Junior College for preparing diketopiperazine of Pro-Hyp. We also acknowledge critical reading of the manuscript by Dr. N. Tominaga of the Ariake National College of Technology.

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